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14. ABSTRACT
In preliminary studies, a potent TG analog (12ADT) was coupled to a series of pentapeptides composed of varying combinations of Asp and Glu to create PSMA-activated prodrugs. One of these prodrugs with the sequence 12ADT-Asp-Glu*Glu*Glu*Glu was efficiently hydrolyzed by PSMA and resulted in accumulation of high levels of the cleaved product in tumor tissue compared to normal tissue. The goal of this study is to take advantage of this selective accumulation of the TG analog to make a prostate cancer specific PSMA targeted imaging agent. Specific Aims: The specific aims of the study are: (1) To synthesize and characterize the cytotoxicity of a series of Iodide labeled Asp- or Glu-containing TG analogs. (2) To synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells. (3) To determine the in vivo efficacy toxicity, pharmacokinetics and biodistribution of 125-I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts; (4) To evaluate added therapeutic efficacy produced by 131-I labeling of the PSMA-activated prodrug in vivo against PSMA producing xenografts. Progress: Over the past year we have developed a 14-step synthesis to generate precursor phenolic TG analog. We documented the analogs ability to bind to the SERCA pump target. We then developed methods to couple the analog to the carrier peptide and confirmed cleavage by PSMA. Finally we developed methods to synthesize and purify the iodinated PSMA-activated agent. This compound is now under evaluation in vivo in biodistribution and imaging studies.

15. SUBJECT TERMS
PSMA, Imaging, Targeted

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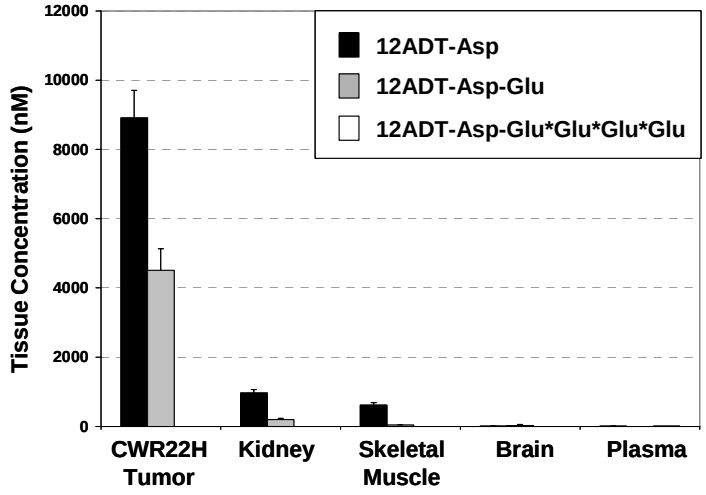
INTRODUCTION:

Our laboratory has been actively engaged in the preclinical development of methods to selectively target a highly potent cytotoxin, thapsigargin (TG), to prostate cancer cells. TG is an abundant natural product that makes up 1% of the weight of the seeds of the umbelliferous plant, *Thapsia garganica*, which grows as a weed throughout the Mediterranean basin. We have been interested in developing TG as a drug on the basis of its ability to kill both proliferating and non-proliferating prostate cancer cells at low nanomolar concentration. Previously our laboratory has demonstrated that prostate cancer cells have a remarkably low rate of proliferation (<5%/day), a finding that, in part, may explain their relatively poor response to standard antiproliferative chemotherapies. In addition, prostate cancers, like most malignancies, are a heterogeneous collection of cells that often express variable amounts of certain target proteins that are not required for cell survival. TG therapy, therefore, could overcome problems of therapeutic resistance due to low proliferative rate and heterogeneity of target expression within prostate tumors because it activates proliferation independent cell death due to its ability to inhibit the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump, a critical intracellular protein whose normal function is required by all cell types for survival. TG inhibition of the SERCA pump results in sustained elevation of intracellular calcium that leads to activation of endonucleases within the nucleus, ER stress responses and release of apoptotic factors by the mitochondria (11). TG's potent cytotoxicity, however, is not prostate cancer specific. Therefore, a strategy must be developed to target TG selectively to prostate cancer cell types while avoiding toxicity to normal, non-proliferating normal host tissues.

Our strategy for targeted TG therapy for prostate cancer has been to inactivate TG's cytotoxicity by coupling it to peptide carriers that are recognized as substrates by prostate tissue specific proteases. Since TG is a highly lipophilic compound that readily partitions into lipid membranes, coupling to a water soluble peptide carrier helps to solubilize TG while at the same time preventing it from passively entering the cell. These TG "prodrugs" can only become activated by release of the peptide by proteases present within sites of prostate cancer. Using this approach we have developed TG prodrugs that are selectively activated by prostate-specific antigen (PSA) (1) and human glandular kallikrein 2 (hK2).

In the course of developing a PSMA-activated TG therapy we identified a prodrug that was readily hydrolyzed by PSMA and selectively toxic to PSMA-producing human prostate cancers in vitro and in vivo. In the course of this work, we began to analyze tissue levels of the TG analog (12ADT-Asp) that is released from the peptide carrier by PSMA, figure 1.

A.



B.

Tissue	%ID/g
Tumor	2.68 ± 0.20
Kidney	0.17 ± 0.014
Skeletal Muscle	0.13 ± 0.15
Brain	0.0076 ± 0.008
Plasma	0.00046 ± 0.0001
Tumor/Kidney	15.4
Tumor/Skeletal Muscle	20.5
Tumor/Brain	354.3
Tumor/Plasma	5816

Figure 1. (A) Selective accumulation of 12ADT-Asp and 12ADT-Asp-Glu in tumor tissue compared to indicated normal tissue five days after single intravenous dose of 2 μmole (120 mg/kg); (B) Biodistribution of TG species (sum of 12ADT-Asp, 12ADT-Asp-Glu and 12ADT-Asp-Glu*Glu*Glu*Glu) in CWR22H tumor bearing mice. Data presented as % Initial dose (ID)/gram and tumor/tissue ratios (n=4 mice).

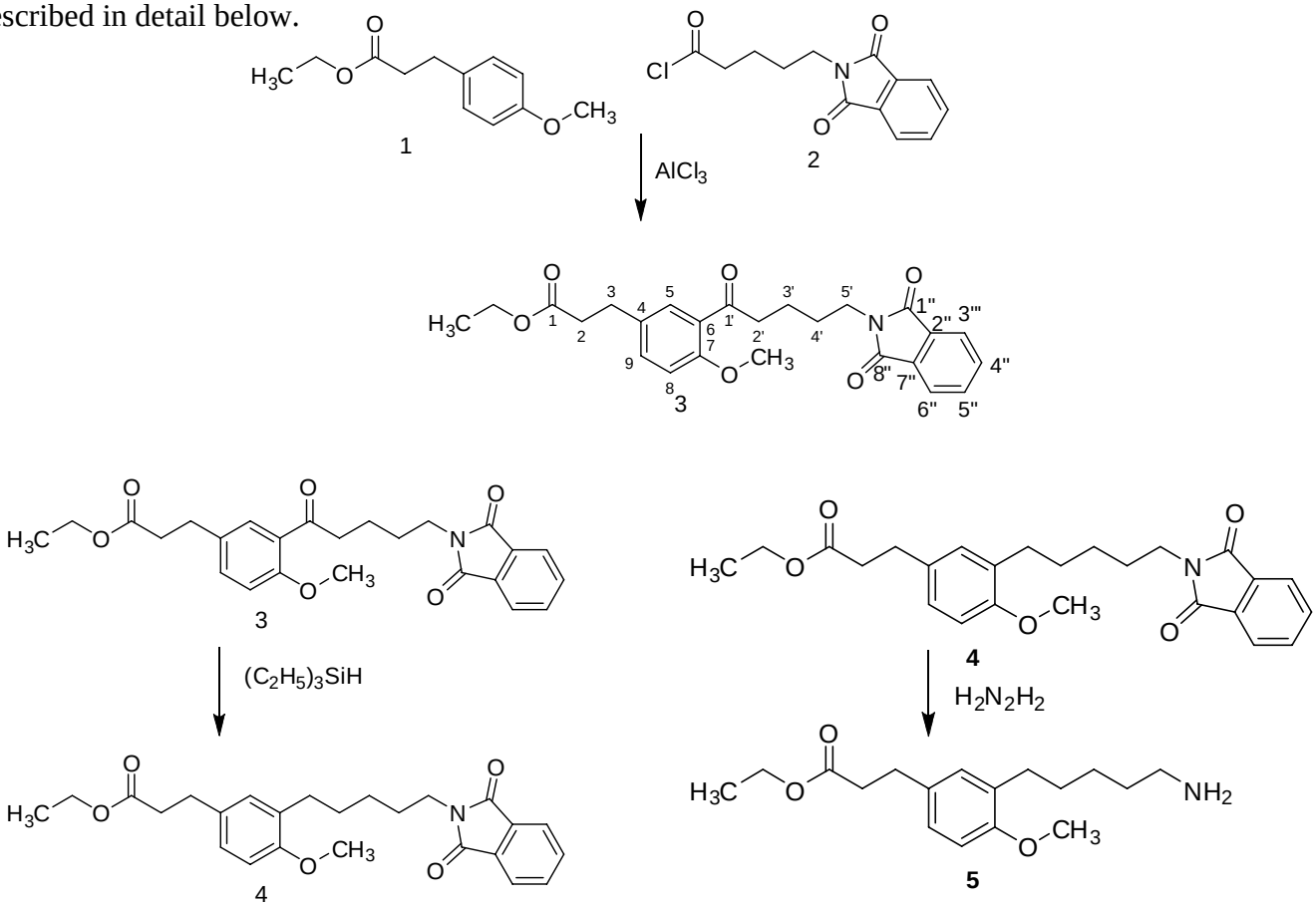
BODY:

Hypothesis: The hypothesis is that a PSMA-Activated Imaging Agent can be achieved by radiolabeling a TG analog that can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). This substrate would be readily cleaved by PSMA within prostate cancer sites resulting in the release of a radiolabeled cytotoxin (thapsigargin analog) that would selectively accumulate in prostate cancer tissue over times. This would then allow PSMA positive prostate cancers to be imaged. As a secondary objective, the cytotoxicity and antitumor efficacy of these PSMA-activated prodrugs will also be evaluated.

Specific Aims: The specific aims of the study are: (1) To synthesize and characterize the cytotoxicity and stability of a series of Iodide labeled Asp- or Glu-containing TG analogs. (2) To synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells in vitro; (3) To determine the in vivo toxicity, pharmacokinetics and biodistribution of 125-I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts.

Progress for Reporting Period 2007-2008.

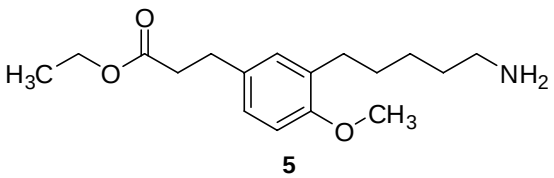
Completion of the first Specific Aim requires the development of a synthetic scheme to generate an TG analog that can be iodinated using standard methods. To accomplish this first step we developed the 14 step synthesis described in detail below.



The first intermediate in this synthesis was Compound **3**. To generate compound **3**, compound **1** (5 g, 24 mmol) was drop wise added to a solution of compound **2** (7.1 g, 26.7 mmol) in CH_2Cl_2 (20 ml). After stirring at room temperature under N_2 for 15 min. $AlCl_3$ (9.9g, 75 mmol) was added portion wise over a period of 10 min. The

reaction mixture, which turned to reddish to orange, was stirred at room temperature under N_2 for 2 h, and water (100 ml) was added. The mixture was adjusted to pH 7 with 5% aqueous $NaHCO_3$, and extracted three times with CH_2Cl_2 (100 ml). The organic phases were combined and concentrated. The residue was purified by flash chromatography using EtOAc/toluene (1:9) as an eluent to give **3** (5g, 66.7%).

Triethylsilane (2.6 g, 5.9 mmol) was added to a solution of **3** (2.6g, 5.9 mmol) in trifluoroactic acid (10 ml) and the solution was stirred for 3 h. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with toluene/ethyl acetate (1: 9) as eluent to give **4** (2 g, 80%). Hydrazine hydrate (120 mg, 2.4 mmol) was added to a stirred solution **4** (1 g, 2.4 mmol) in 15 ml of MeOH. After stirring for 3 h 15 ml of NH_4OH.were added, and the mixture was extracted three times with CH_2Cl_2 (20 ml). The organic phases were combined and concentrated to give the **5** (650 mg, 94%).



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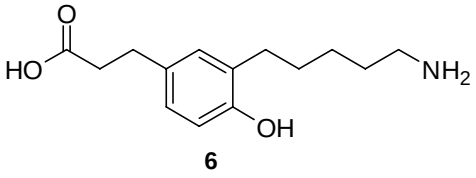
HBr



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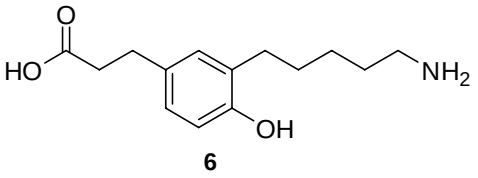

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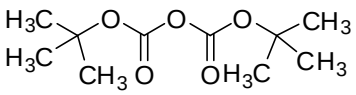

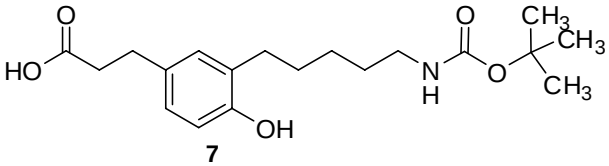

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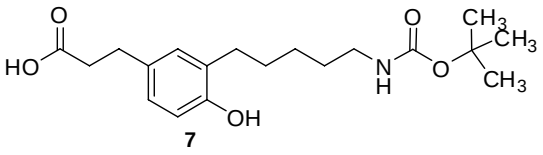
The crude compound **6** was dissolved in 10 ml of 48% aqueous HBr. The reaction mixture was heated to reflux for 4 h and added iced water (50 ml). The aqueous solution was washed twice with ether (50 ml) and the aqueous phase concentrated to give **6** (600 mg). The crude **6** (600 mg, 2.4 mmol) was dissolved in acetone (7.5 ml) and water (2.5 ml) and to the solution was added added $NaHCO_3$ (900 mg) and Boc_2O (900 mg, 4.1 mmol). The reaction mixture was stirred for 2 h and concentrated in vacuo to half volume and the precipitate removed by filtration. The filtrate was concentrated in vacuo and the residue chromatographed on RP18 to give **7** (500 mg, 51%) using methanol-water (5:1) added 1% of acetic acid as an eluent.



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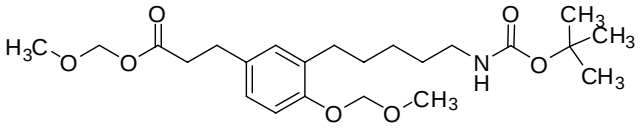

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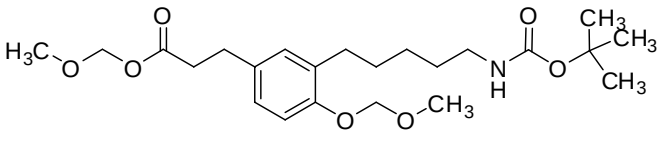

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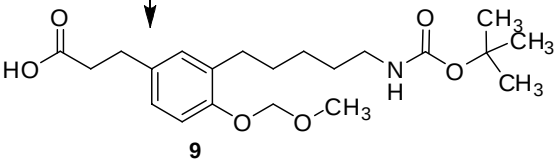
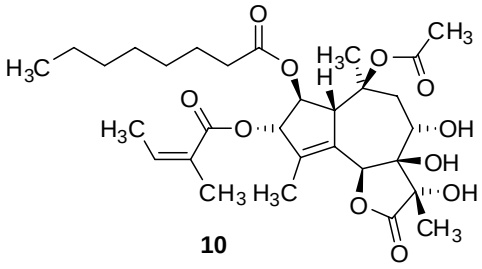


9

Chloromethyl methyl ether (230 mg, 2.9 mmol) and N,N-diisopropylethylamine (370 mg, 2.9 mmol) were added to a refluxed solution of **7** (500 mg, 1.7 mmol) in 20 ml of acetonitrile. After reflux for 30 min three additional protions of chloromethyl methyl ether (each times 230 mg) and N,N-diisopropylethylamine (each times 370 mg) followed by reflux for additional 30 min after each addition. After reflux for 30 min after

addition of the fourth portions of reagents the mixture was cooled to room temperature and added water (30 ml) and a saturated aqueous solution of $NaHCO_3$ (20 ml). The reaction mixture was extracted three times with ether (20 ml). The extracts were concentrated in vacuo. The residue was purified by CC to give compound **8** (220 mg, 30%).

Lithium hydroxide hydrate (100 mg) was added to a solution of **8** (400 mg) in 10 ml of methanol-water (1:1). The mixture was stirred for 3h at room temperature and concentrated in vacuo. The residue was dissolved in 10 ml of water and acidified with hydrochloric acid (6 M) to pH 2. The mixture was extracted three times with CH_2Cl_2 (20 ml). The combined organic phases were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to give compound 11 (300 mg, 92%)



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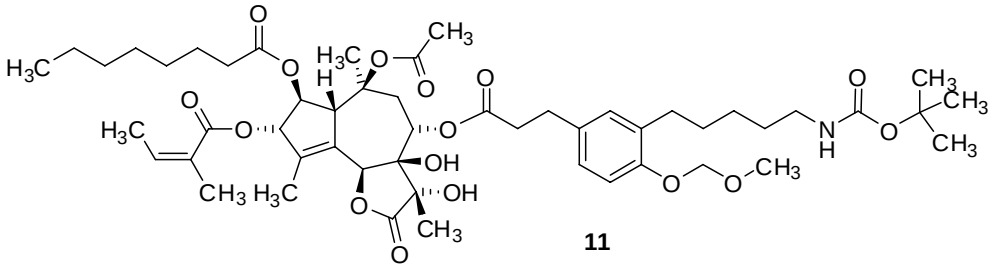
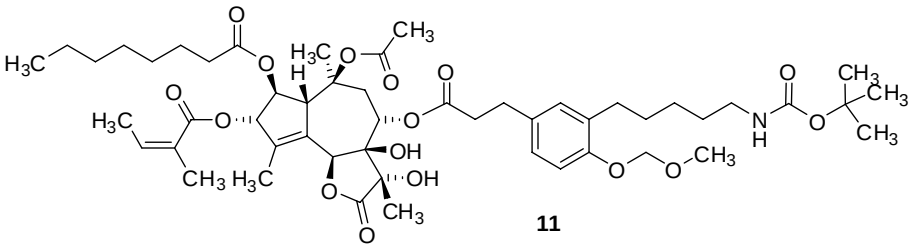


11

Dicyclohexylcarbodiimide (230 mg, 1.1 mmol) was added to a solution of **9** (400mg 1.1 mmol), DBTg (**10**) (607 mg, 1.1 mmol), and DMAP (50 mg) in dry CH_2Cl_2 (5 ml). After stirring for 4 h at room temperature the reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was purified by Chromatography on RP18 using CH_3OH/H_2O (4:1) added 0.1% AcOH as eluent to give compound 12 (650 mg, 55%).



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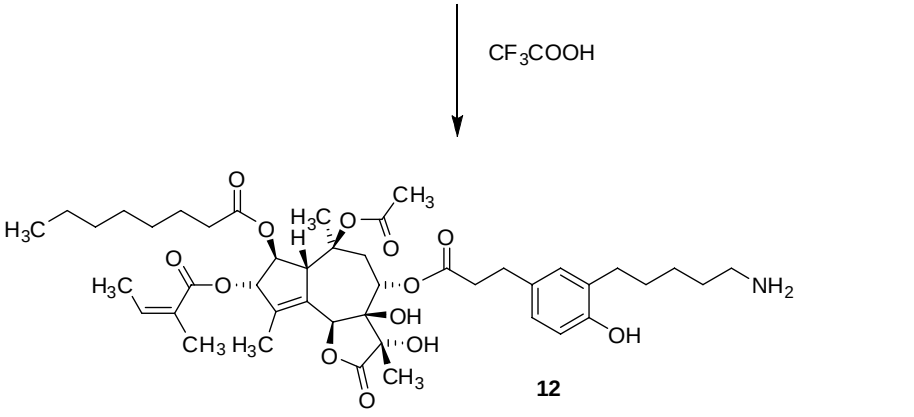
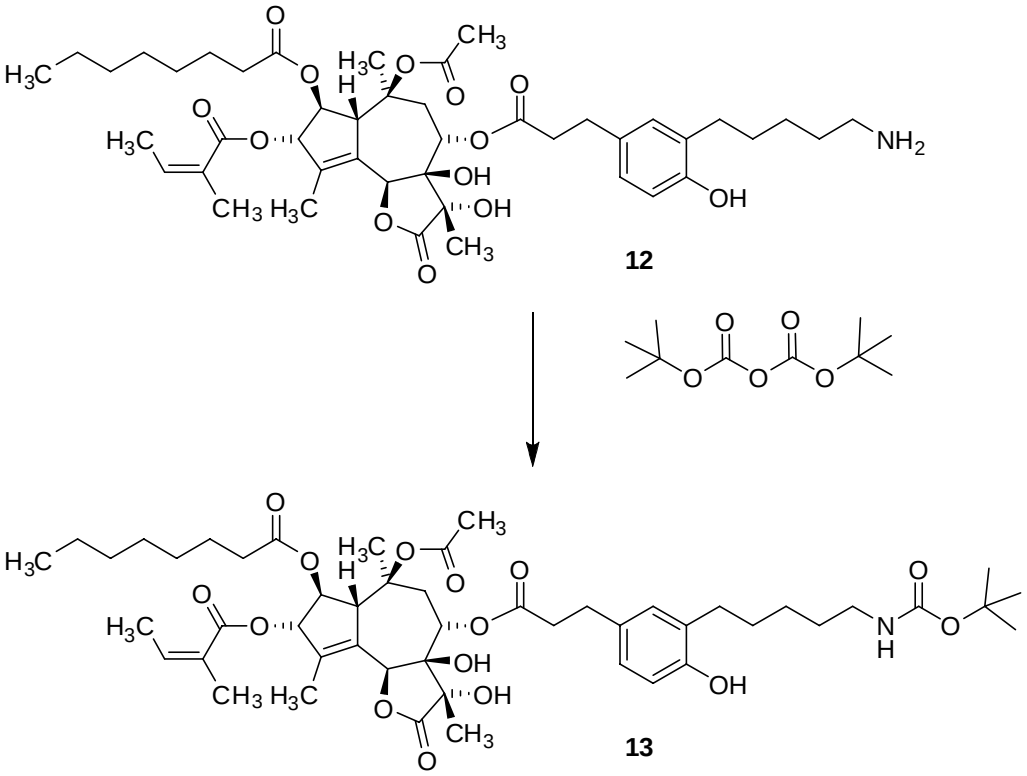
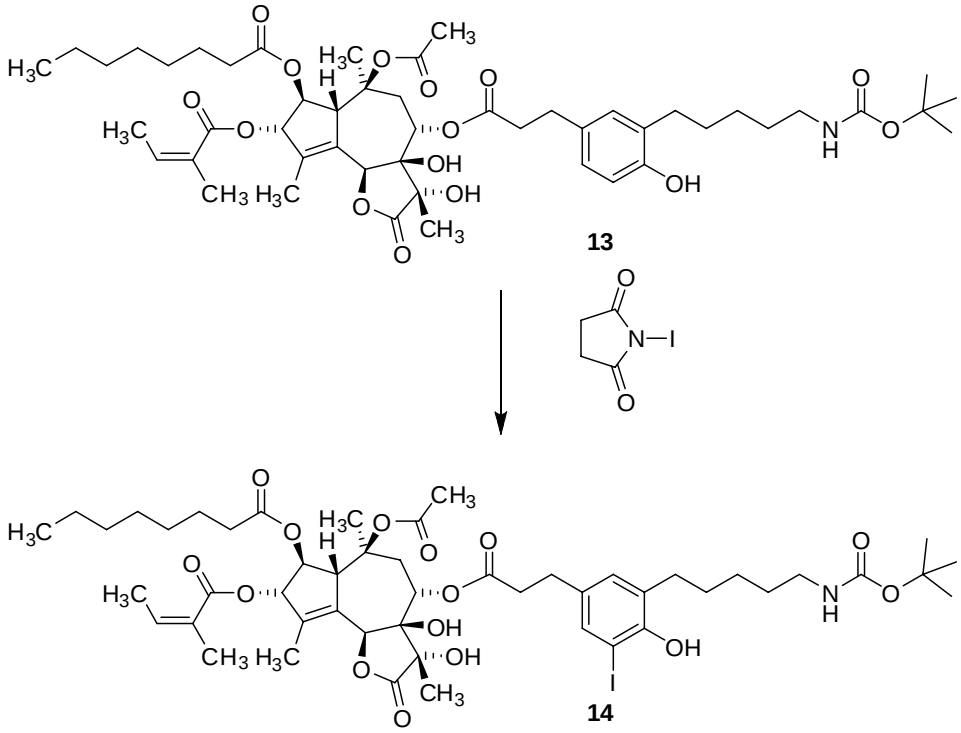


12

Trifluoroacetic acid (2 ml) was added to a solution of **11** (300 mg, 0.31 mmol) in dichloromethane (6 ml) and the mixture was stirred for 3 h. The solution was concentrated in vacuo to give **12**.



Di-*tert*-butyldicarbonate (50 mg, 0.2 mmol) and DMAP (50 mg) was added to a solution of **12** in dichloromethane (4 ml) and the mixture was stirred for 30 min. The residue after concentration was fractionated by CC over RP18 using methanol-water (5:1) added 1% of glacial acetic acid as an eluent to give **12** (130 mg, 46%).



N-Iodosuccinimide (35 mg, 0.2 mmol) and *p*-toluenesulfonic acid (5 mg, 0.03 mmol) was added to a solution of **13** (70 mg, 0.1 mmol) in dry dichloromethane and the mixture was stirred for 1.5 h. A solution of aqueous

sodium thiosulfate (10%, 10 ml) was added and the mixture stirred for additional 10 min. The aqueous phase was isolated and extracted twice with dichloromethane (10 ml). The combined organic phases were concentrated and the residue fractionated by CC over RP18 using methanol-water (5:1) added 1% of glacial acetic acid as an eluent to give **14** (56 mg, 70%).

All materials were purified by column chromatography with structure confirmed by NMR analysis and mass spectroscopy. The purified phenolic TG analog 14 was then tested in a previously described microsomal assay system to assess inhibition of the SERCA pump compared to TG, figure 2. In this assay, compound 14 was ~ 4-fold less potent than TG but still maintained potent ability to inhibit the SERCA pump at nanomolar concentrations.

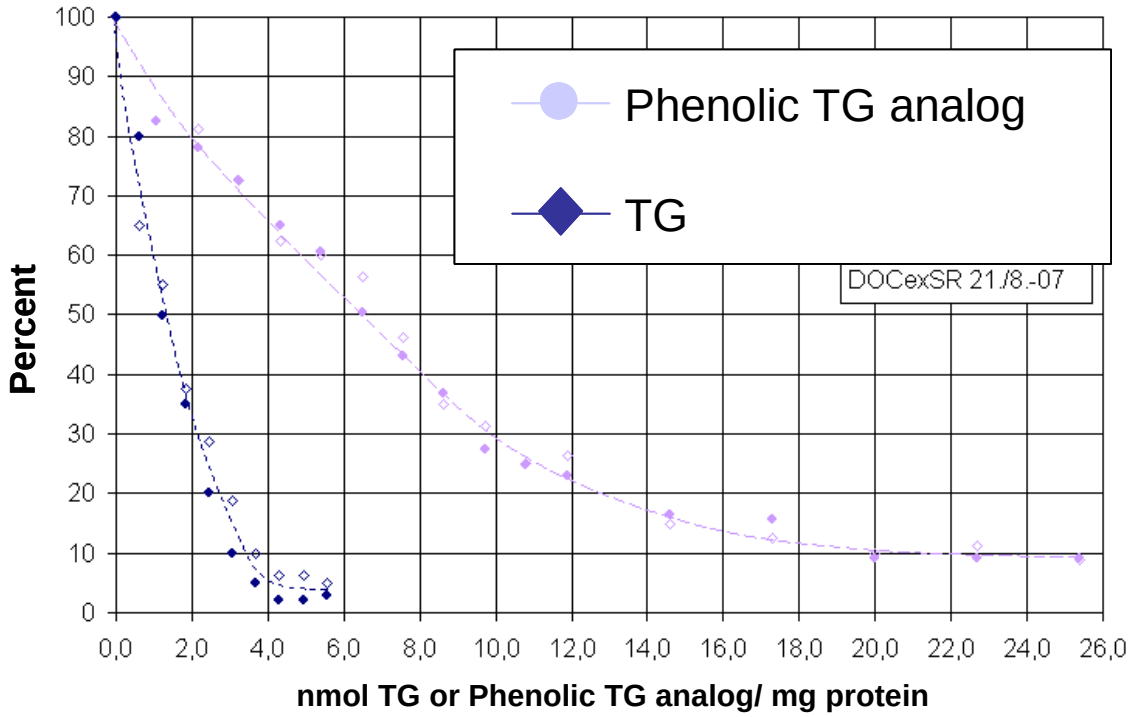


Figure 2. Microsomal assay comparing inhibition of SERCA pump by compound 14 and TG over a range of concentrations.

Progress was also made on Specific Aim 2 where the goal was to synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells in vitro. In initial studies we evaluated the hydrolysis of the non-iodinated PSMA prodrug (PD-14) by PSMA producing LNCaP human prostate cancer cells in vitro. In this assay we saw cleavage of the phenolic TG analog-Asp-Glu-Glu-Glu-Glu prodrug to two forms, phenolic TG (Ph12ADT) and the Asp-Ph12ADT (D-Ph12ADT) consistent with PSMA hydrolysis of the gamma linked glutamate residues, figure 3. Both analogs were observed in the conditioned media and in the cells, consistent with ability of the analogs to penetrate cell membrane once liberated from the peptide carrier. In contrast, no intact PD-14 was observed in the cell extract, consistent with the ability of the highly charged carrier peptide to keep the agent out of cells.

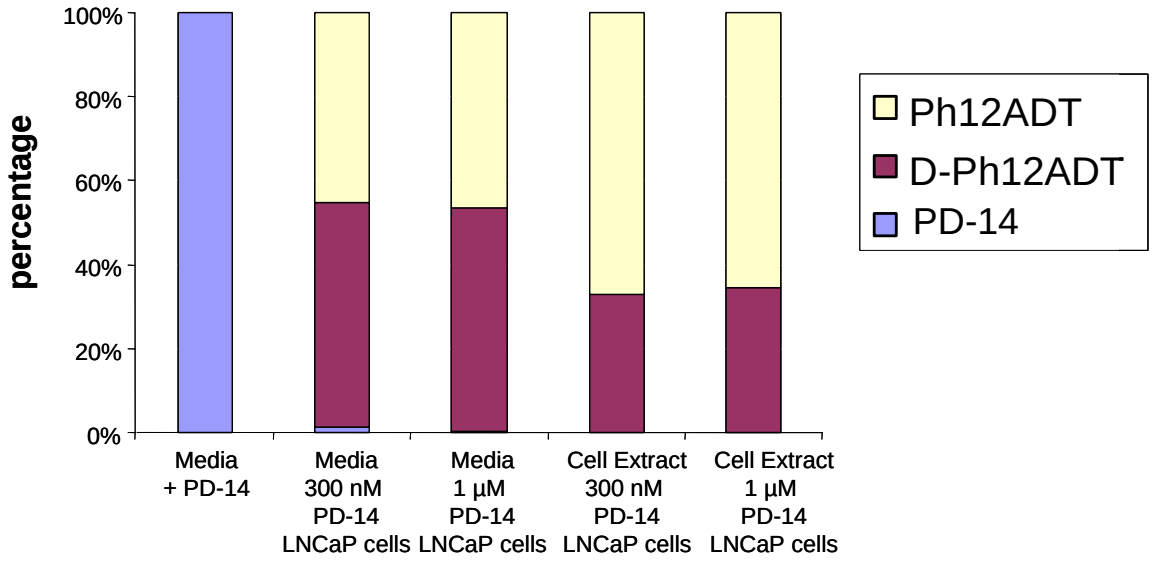


Figure 3. Relative Hydrolysis of PD-14 by LNCaP cells. LNCaP cells (confluent) were treated with media and PD-14 for 3 days. After 3 days the media was collected and the cells were scraped and extracted with acetonitrile. The media and the cell extract were run on the LCMS.

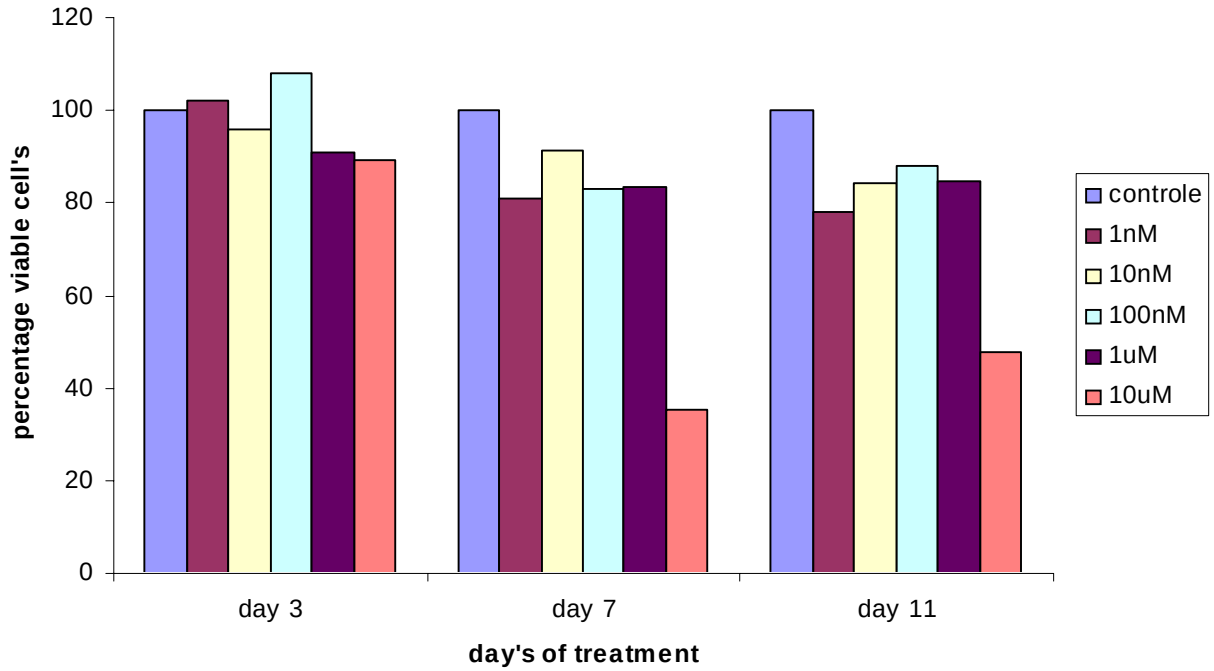


Figure 4. MTT assay evaluating varying concentrations of I-PD-14 against PSMA producing LNCaP cells.

Subsequent we developed methods to iodinate PD-14 and to purify to homogeneity. Evaluation of I-PD-14 for cytotoxicity against PSMA producing cell in tissue culture revealed toxicity of the compound at the relatively high concentration of 10 µM, figure 4. This result suggests that, while PD-14 is cleaved as evidenced by cleavage data in figure 3, insufficient TG analog is released at concentrations < 10 µM to produce a cytotoxic effect.

Key Research Accomplishments

- Developed a 14 step method to synthesize a radioiodinateable TG analog
- Developed methods to synthesize a PSMA-cleavable prodrug
- Synthesized 25 mg of PSMA prodrug precursor for iodination
- Demonstrated cleavage of PSMA prodrug by PSMA producing prostate cancer cells
- Documented cytotoxicity of the PSMA prodrug
- Developed methods to synthesize and purify 125-I labeled PSMA prodrug

Reportable Outcomes

- Presentations: "PSMA-Activated Imaging Agents" Presented at Tri-Institutional SPORE meeting Newport, RI, 2008
- Training: Provided support for training program of Post-Doctoral Fellow J. Michiel Sedelaar
- Grant Application: "PSA-Targeted Combined Radio-Chemotherapy for Prostate Cancer" DOD Synergy Award application 2007 based on preliminary data generated in this award. Status: Not Funded.

Conclusion

The goal of these studies is to develop a PSMA-Activated Imaging Agent by radiolabeling a TG analog that can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). This substrate would be readily cleaved by PSMA within prostate cancer sites resulting in the release of a radiolabeled cytotoxin (thapsigargin analog) that would selectively accumulate in prostate cancer tissue over times allowing PSMA positive prostate cancers to be imaged. Over the first year of funding we have made significant progress to achieving this goal. The major accomplishment of the first year of funding has been to develop a synthetic method to generate phenolic ring containing TG analogs that can be readily iodinated. The synthetic scheme has the flexibility to allow us to move the ring to different positions within the side chain of the TG analog. Using this method, additional TG analogs will be similarly synthesized and characterized over the next year of funding. In addition to the synthesis of the TG analog, we have also developed method to couple the non-iodinated analog to the peptide carrier. Once this prodrug is produced we developed a method to rapidly iodinate the peptide-TG analog and worked out method of separating trace ^{125}I labeled final product from starting material to yield highly purified compound that will be tested over the ensuing year for biodistribution, toxicity and imaging potential

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